THE AMINO ACID SEQUENCE OF ADZUKI BEAN PROTEINASE INHIBITOR I

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1. Introduction

Proteins inhibiting the activity of proteinases are widely distributed in plant and animal tissues, and they are especially rich in leguminous seeds [1]. Knowledge of the amino acid sequences of these proteinase inhibitors is important for studies on their structure–function relationships and molecular evolution. As for legume inhibitors, the amino acid sequences of Bowman-Birk soybean inhibitor [2] and two additional soybean inhibitors [3] have been determined completely, and those of lima bean inhibitors IV and IV′ [4] and garden bean inhibitors II and II′ [5] nearly completely. These results show that these legume inhibitors are a family of low molecular weight proteins composed of about 70–80 amino acid residues and are double-headed, i.e., consisting of two domains each having a proteinase reactive site.

Here, we report the amino acid sequence of adzuki bean proteinase inhibitor I, one of the major proteinase inhibitors isolated from adzuki bean (*Phaseolus angularis*) 'Takara'. This inhibitor is obtained in a crystalline form and is also double-headed, inhibiting trypsin and chymotrypsin simultaneously and independently like lima bean inhibitor IV and Bowman-Birk soybean inhibitor, as will be detailed elsewhere. A similar double-headed inhibitor, inhibiting both trypsin and chymotrypsin, has also been isolated independently from adzuki bean 'Dainagon' [6].

2. Materials and methods

2.1. *Preparation of adzuki bean proteinase inhibitor I*

The inhibitor was extracted from adzuki bean 'Takara' as in [7] with a slight modification, and purified by a combination of chromatographies on SP–Sephadex, DEAE-cellulose and Sephadex G-75. The purified material was homogenous as examined by polyacrylamide disc-gel electrophoresis both with and without sodium dodecyl sulfate. The details of the purification will be described elsewhere.

2.2. *Cyanogen bromide cleavage and isolation of peptides*

The inhibitor was reduced and carboxymethylated according to [8] then treated with cyanogen bromide in 70% formic acid at 40°C for 32 h. The cyanogen bromide peptides were fractionated on a Sephadex G-50 column (1.5 × 210 cm) equilibrated and eluted with 0.1 M ammonium bicarbonate (pH 7.8).

2.3. *Enzymatic digestions and purification of peptides*

Peptides were digested in 0.1 M ammonium bicarbonate (pH 7.8) at 37°C with TPCK-treated trypsin (Worthington) for 4 h (peptide/enzyme wt ratio, 50/1), with α-chymotrypsin (Worthington) for 8 h (peptide/enzyme wt ratio, 50/1) or with *Staphylococcus aureus* protease (Miles) [9] for 40 h (peptide/enzyme molar ratio, 10/1). The resulting peptides were purified by chromatography on a Sephadex G-25 (superfine) column (1.5 × 210 cm) and/or high-voltage paper electrophoresis, at pH 3.5 and/or 6.4.

2.4. *Amino acid analyses and sequencing*

Amino acid analyses were performed with a Jeol 6AH amino acid analyzer after hydrolysis with

6 N HCl at 110°C for 24 h in sealed, evacuated tubes (data not shown). The amino acid sequence analyses were performed by a modification [10] of the manual Edman degradation method [11]. PTH-amino acids were identified by thin-layer chromatography on polyamide layer sheets [12] and/or by high-performance liquid chromatography on Partisil 10 columns (0.4 × 25 cm) using dichloromethane–dichloroethane (1:1) for apolar PTH-amino acids and dichloromethane/methanol/dimethylsulfoxide/acetic acid (94:3.4:2.1:0.013) for polar PTH-amino acids. Dansylation method [13,14] was also used for the end-group analyses and in some cases was applied to sequence analysis.

3. Results and discussion

Figure 1 shows the complete amino acid sequence of adzuki bean inhibitor I. The amino acid analysis of this inhibitor showed the presence of only 1 residue methionine/molecule protein. Therefore, cyanogen bromide treatment of the reduced-carboxymethylated inhibitor yielded two peptide fragments, CB · I and CB · II comprizing the C-terminal portion (residues 29–82) and the N-terminal portion (residues 1–28), respectively, of the protein. The N-terminal peptide, CB · II, was digested separately by trypsin and by *Staphylococcus aureus* protease. Papar electrophoresis of the tryptic digest gave two peptides, CB · II–T · 1

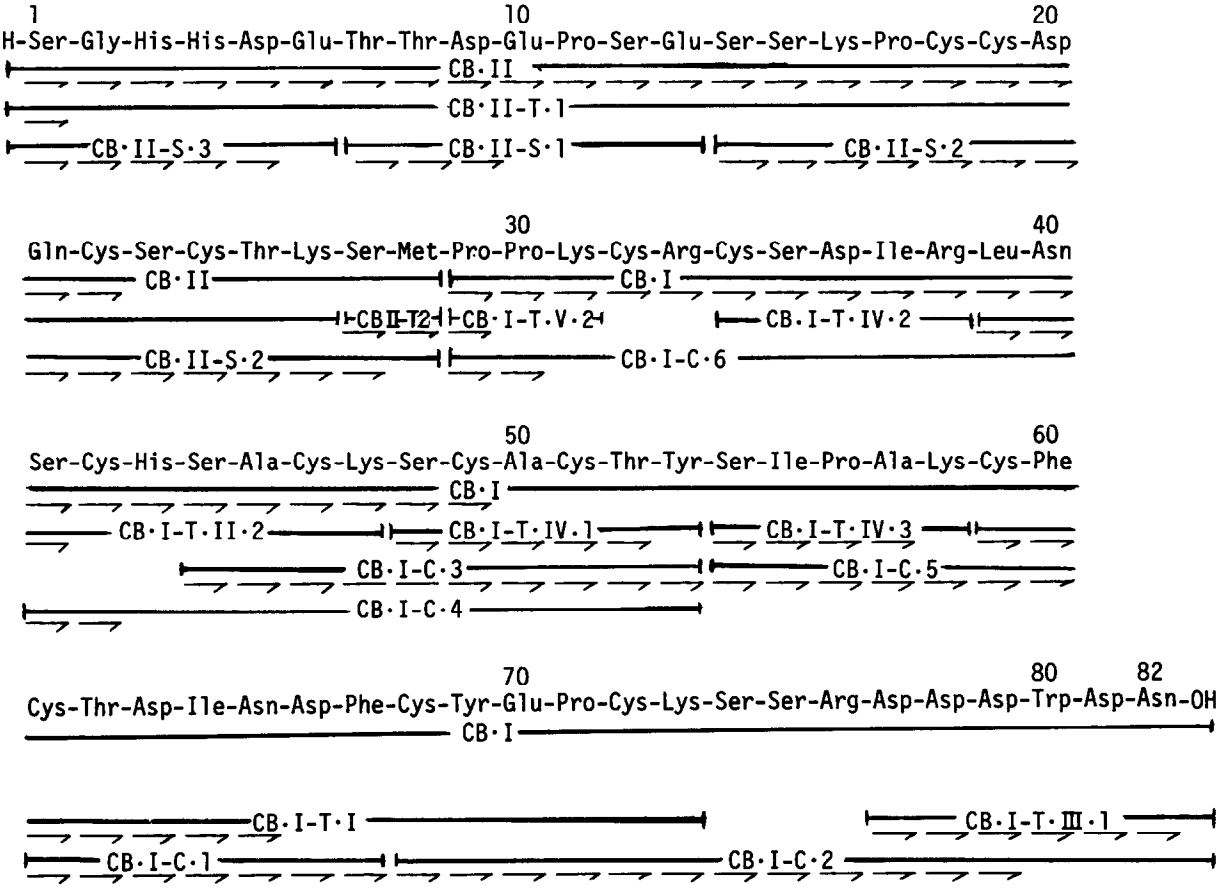

Fig.1. The amino acid sequence of adzuki bean proteinase inhibitor I. Arrows indicate the residues identified by manual Edman degradation. CB, S, T and C stand for fragments obtained by cleavages with cyanogen bromide, *Staphylococcus aureus* protease, trypsin and α-chymotrypsin, respectively.

and CB · II–T · 2, and that of the *Staphylococcus* protease digest, three peptides, CB · II–S · 1,2 and 3. Sequence analysis of these peptides established the amino acid sequence of CB · II. On the other hand, the C-terminal peptide, CB · I, was digested separately by trypsin and by α-chymotrypsin. The tryptic peptides were fractionated by Sephadex G-25 chromatography to give five peptide fractions, CB · I–T · I–V, which were further purified by paper electrophoresis to give finally seven major peptides. The chymotryptic peptides were fractionated directly by paper electrophoresis to yield six peptides. Sequencing of these peptides established the amino acid sequence of CB · I.

Thus the complete amino acid sequence of adzuki bean inhibitor I was deduced as shown in fig.1. The total number of the residues in the protein was found to be 82, which fully accounts for the composition of the protein. Based on this composition (Asp 10; Asn 3; Thr 5; Ser 13; Glu 4; Gln 1; Pro 6; Gly 1; Ala 3; Cys 14; Val 0; Met 1; Ile 3; Leu 1; Tyr 2; Phe 2; Trp 1; Lys 6; His 3; Arg 3), mol. wt 9116 is calculated.

The amino acid sequence of adzuki bean inhibitor I is compared with those of other typical legume double-headed inhibitors in fig.2. A very high homology is obvious among these inhibitors. The amino acid sequence of adzuki bean inhibitor I resembles especially that of lima bean inhibitors. This is in accord with the fact that adzuki bean and lima bean are most closely related species in the classification of legumes [15]. The sequence comparison also indicates that Lys 26–Ser 27 and Tyr 53–Ser 54 are proteinase reactive sites. Since adzuki bean inhibitor I is double-headed, inhibiting trypsin and chymotrypsin simultaneously, it seems reasonable to conclude that Lys 26–Ser 27 is the trypsin reactive site, and Tyr 53–Ser 54, the chymotrypsin reactive site. The latter finding is especially interesting since only Leu–Ser and Phe–Ser have so far been known as the chymotrypsin reactive sites of legume double-headed inhibitors. It is also of interest that the Tyr 53–Ser 54 bond was very easily cleaved upon digestion of CB · I by TPCK-treated trypsin. The presence of one residue of tryptophan is notable since this residue is absent in most legume inhibitors.

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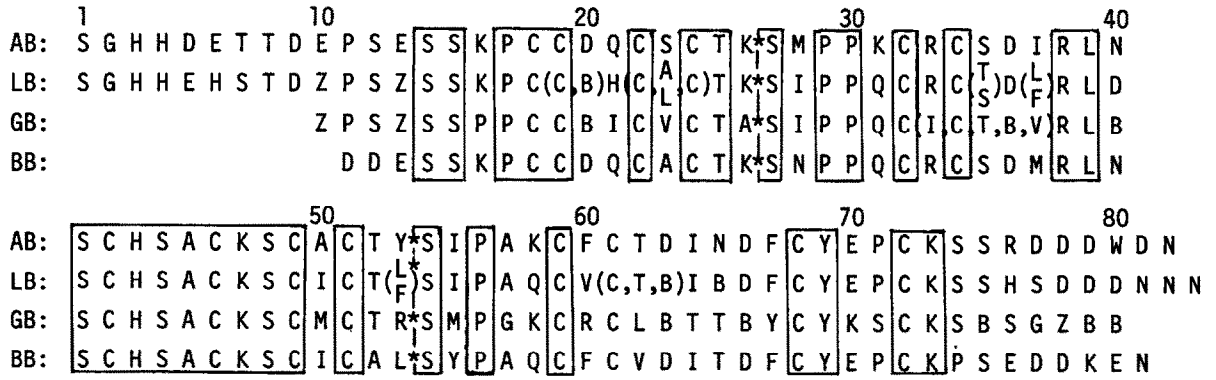

Fig.2. Comparison of the amino acid sequence of adzuki bean inhibitor I with those of other typical legume double-headed inhibitors. Amino acid residues are expressed in the single letter code: A (Ala), B (Asx), C (Cys), D (Asp), E (Glu), F (Phe, G (Gly), H (His), I (Ile), K (Lys), L (Leu), M (Met), N (Asn), P (Pro), Q (Gln), R (Arg), S (Ser), T (Thr), V (Val), W (Trp), Y (Tyr), Z (Glx). Enclosed amino acid residues are common to all four inhibitors. The reactive sites are shown by asterisks. AB, adzuki bean inhibitor I; LB, lima bean inhibitor IV and IV′; GB, garden bean inhibitor II′; BB, Bowman-Birk soybean inhibitor.

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